Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Enhancement of Toll-like receptor 2-mediated immune responses by AIMP1, a novel cytokine, in mouse dendritic cells

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doi:10.1111/j.1365-2567.2011.03468.x
Received 20 January 2011; revised 27 May 2011; accepted 31 May 2011.
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Summary

Aminoacyl tRNA synthetase-interacting protein 1 (AIMP1) is a novel pleiotropic cytokine that was identified initially from Meth A-induced fibrosarcoma. It is expressed in the salivary glands, small intestine and large intestine, and is associated with the innate immune system. Previously, we demonstrated that AIMP1 might function as a regulator of innate immune responses by inducing the maturation and activation of bone-marrow-derived dendritic cells (BM-DCs). Toll-like receptors (TLRs) are major pathogen-recognition receptors that are constitutively expressed on DCs. In this study, we attempted to determine whether AIMP1 is capable of regulating the expression of TLRs, and also capable of affecting the TLR-mediated activation of DCs. Expression of TLR1, -2, -3 and -7 was highly induced by AIMP1 treatment in BM-DCs, whereas the expression of other TLRs was either down-regulated or remained unchanged. In particular, the expression of the TLR2 protein was up-regulated by AIMP1 in a time-dependent and dose-dependent manner, and was suppressed upon the addition of BAY11-7082, an inhibitor of nuclear factor-κB. AIMP1 was also shown to increase nuclear factor-κB binding activity. Importantly, AIMP1 enhanced the production of interleukin-6 and interleukin-12, and the expression of co-stimulatory molecules on BM-DCs when combined with lipoteichoic acid or Pam3Cys, two well-known TLR2 agonists. Collectively, these results demonstrate that the AIMP1 protein enhances TLR2-mediated immune responses via the up-regulation of TLR2 expression.

Keywords: aminoacyl tRNA synthetase-interacting protein 1; cytokines; dendritic cells; rodent; Toll-like receptor 2; Toll-like receptor 2 agonist

Introduction

The innate immune system functions as a sentinel, and contributes to the eradication of pathogens and the establishment of adaptive immunity. Pattern-recognition receptors play a critical role in distinguishing the invading microorganisms.[1] Among pattern-recognition receptors, Toll-like receptors (TLRs) are characterized by their potent immune-adjuvant activity to activate dendritic cells (DCs), macrophages and other antigen-presenting cells. Recently, it has been determined that TLRs include 10 (TLRs 1–10) and 12 (TLRs 1–9 and 11–13) family members in human subjects and mice, respectively.[2] The best-studied family members are TLR2 and TLR4, which are mainly expressed on the surfaces of monocyte/macrophage and neutrophil lineages;[3] TLR2 detects the components of Gram-positive bacteria such as lipoteichoic acid (LTA), and of all bacteria such as lipoprotein, whereas TLR4 detects the bacterial cell-wall component lipopolysaccharide (LPS).[4] Interaction with their ligands ultimately leads to the activation of the transcription factor nuclear factor-κB (NF-κB) through adaptor molecules such as TIRAP and MyD88, and induces the expression of inflammatory cytokine genes.[3]

Aminoacyl tRNA synthetase-interacting protein 1 (AIMP1) is a non-enzymatic component complexed with aminoacyl tRNA synthetase and is also known as p43 or pro-EMAP II, which is converted into EMAP II by proteolytic cleavage. The C terminus of AIMP1 is identical to that of EMAP II.[5] This protein is composed of 312 amino acids and is determined as having a molecular weight of 43 000. AIMP1 has dose-dependent biphasic activities on

angiogenesis and anti-cancer effects.[6,7] It is secreted from immune cells (macrophages), prostate cancer cells and transfected cells (293 cells transfected with pcDNA-p43).[8–10] The secreted AIMP1 can function as a regulator of innate immune responses by inducing the maturation and activation of bone-marrow-derived dendritic cells (BM-DCs).[11] The AIMP1 protein is expressed in several organs including the salivary gland, small intestine and large intestine, and is related to the innate immune system.[12] In particular, the expression of AIMP1 is up-regulated in LPS-induced acute lung inflammation. This up-regulated AIMP1 expression is detected in monocytes/macrophages at 1 and 3 hr after LPS challenge.[13]

We here demonstrate that AIMP1 may perform additional functions in the induction of DC activation via the up-regulated expression of TLR2. AIMP1 significantly up-regulates TLR2 expression levels via increased NF-κB binding activity at the TLR2 promoter region. Moreover, AIMP1 may enhance LTA-induced cytokine production and the expression of surface molecules.

Materials and methods

Experimental animals

Female 8- to 10-week-old C57BL/6 mice were purchased from OrientBio, Inc. (Seoul, Korea). The mice were maintained under specific viral pathogen-free conditions and were treated according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Monoclonal antibodies, cytokines and reagents

Recombinant murine interleukin-6 (IL-6) and IL-10 were obtained from Peprotech (Rocky Hill, NJ). Anti-IL-6 (20F3.11 and 32C11.4), anti-IL-10 (2A5 and SXC2), anti-IL-12p70 monoclonal antibodies (mAbs), and recombinant IL-12p70 were purchased from eBioscience (San Diego, CA). Blocking antibodies to TLR2 (clone T2.5; mouse IgG1) and TLR4 (clone MTS510; rat IgG2a), and mouse IgG1 were purchased from eBioscience. The LPS (from *Escherichia coli* 0111:B4), LTA (from *Staphylococcus aureus*) and Pam3Cys were purchased from Sigma (St. Louis, MO).

Expression and purification of AIMP1

The AIMP1 (312 amino acids) was expressed as a His tag fusion protein in *Escherichia coli* BL21 (DE3) and purified via nickel affinity chromatography and Mono Q or S ion-exchange chromatography. To remove endotoxins such as LPS, the protein solution was dialysed in pyrogen-free buffer (10 mM potassium phosphate buffer, pH 6·0, 100 mM NaCl). After dialysis, the AIMP1 solution was loaded onto polymyxin resin (Bio-Rad, Hercules, CA), pre-equilibrated with the same buffer, incubated for 20 min, and then eluted. To further remove residual LPS, the protein solution was dialysed against PBS containing 20% glycerol and filtered with a Posidyne membrane (Pall Gelman Laboratory, Ann Arbor, MI). The concentration of the LPS in AIMP1 was < 0·05 endotoxin units/10 μg protein (where 1 endotoxin unit = 0·1 ng/ml *E. coli* LPS), as determined using the *Limulus* Amoebocyte lysate QCL-1000 kit (BioWhittaker, Walkersville, MD).

Generation of bone marrow-derived dendritic cells

The BM-DCs were generated via a modified version of the method originally described by Inaba *et al.*[14] In brief, the femurs and tibiae of mice were removed and the marrow was flushed with ice-cold RPMI-1640 with a syringe equipped with a 26-gauge needle. Larger cell clusters were dissociated by gentle pipetting and the cell suspension was filtered through a 70 μm nylon cell strainer (BD Falcon, Bedford, MA). Red blood cells were lysed with a lysing solution containing 0·15 M NH_4Cl, 1 mM $KHCO_3$ and 0·1 mM EDTA. The bone marrow cells were then suspended in growth medium. The number of cells in the suspension was then adjusted to 5×10^5 cells/ml, and the cell suspension was added to the culture dishes. The cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (Biomeda, Foster City, CA), 50 μM β-mercaptoethanol (Sigma), 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Rockville, MD) supplemented with 10 ng/ml of granulocyte–macrophage colony-stimulating factor (ProSpec, Rehovot, Israel). The culture medium containing cytokine was replaced on day 3. On day 5, the non-adherent cells were removed, and fresh medium containing cytokine was added. On day 6 or day 7 of culture, non-adherent cells, and loosely adherent dendritic cell aggregates were harvested and the percentage of $CD11c^+$ cells (> 94%) was checked by fluorocytometric analysis (BD Biosciences, San Jose, CA) before their use in the experiments (see Supplementary material, Fig. S1).

Reverse transcriptase–polymerase chain reaction

Total RNA obtained from the cells was reverse-transcribed into cDNA, and then PCR amplification of the cDNA was conducted using an MJ thermal cycler (Bio-rad, Watertown, MA). The sequences of the PCR primers used in this study were as follows: TLR2 [forward (F)] GTGGAGACACAGCTTAAAGG; TLR2 [reverse (R)] CAGCCTGA GACACTTAGACC; TLR4 (F) CAGCCTGA GACACTTAGACC; TLR4 (R) TGAAGTCTATGGAGGG TGTC. After amplification, the products were separated on 1·5% (weight/volume) agarose gels and stained with ethidium bromide.

Western blot analysis

Dendritic cells were plated (4×10^6 cells) in 12-well dishes, after which the indicated concentrations of AIMP1 were added for the indicated times. The cells were harvested and lysed in lysis buffer (50 mM Tris–HCl, pH 8·0, 150 mM NaCl, 1·0% Nonidet P-40, 0·5% sodium deoxycholate, 0·1% SDS, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml chymostatin, 5 μg/ml leupeptin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM β-glycerophosphate), and the lysates were centrifuged for 15 min at 20 000 ***g***. β-Glycerophosphate, a protease inhibitor, was included in the lysis buffer to prevent AIMP1 proteolysis by proteinase(s) which might be present during the processes. The cell lysates were then separated via 10% SDS–PAGE before being transferred to nitrocellulose membranes. The membranes were then incubated with washing buffer (PBS containing 0·1% Tween-20) containing 2% skim-milk for at least 1 hr, to block any non-specific protein binding. Primary mAb was diluted to 1/5000 in washing buffer, and applied for 2 hr to the membrane at room temperature. After washing, the blots were incubated with the appropriate biotin-conjugated secondary mAb (diluted to 1/5000 in washing buffer) for 1 hr at room temperature followed by incubation with horseradish peroxidase–streptavidin (diluted to 1/10 000 in washing buffer). The immunoreactive bands were visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech Inc, Piscataway, NJ) and imaged on a Fuji LAS-3000 system (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Flow cytometric analysis of cell surface phenotype

Dendritic cells (1×10^6 cells) were harvested, washed with PBS and resuspended in FACS washing buffer (0·5% fetal bovine serum and 0·05% sodium azide in PBS). The cells were then stained with FITC-conjugated anti-CD11c and phycoerythrin-conjugated antibodies as follows: anti-I-A^b, anti-CD40, anti-CD86, anti-CD80 and anti-TLR2. The stained cells were analysed in a FACScan cytometer (BD Biosciences). Data were analysed using CellQuest software (BD Immunocytometry Systems, San Jose, CA).

Electrophoretic mobility gel shift assay

The nuclear extracts were prepared from the cells as described previously.[15] Protein concentrations were determined via a bicinchoninic acid protein assay (Pierce, Rockford, IL). The nuclear extracts were either immediately assayed or stored at −70° until further use. Oligonucleotides containing NF-κB-binding sites within the TLR2 promoter region were employed as probes. The sequences of the probes used in this study were as follows: NF-κB#1 (F) GGCCCTGACCTGGGGACATCCCCTTCCCTC; NF-κB#1 (R) GGAAGTGAGGGA AGGGGATGTCCCCAGGTC; NF-κB#2 (F) CTTTAGGACACCTGGGGAATT CCCACACG G; NF-κB#2 (R) GAGGCTCCGTGTGGGAATTCCCCAG GTGTC. The oligonucleotides were end-labelled with [α-^{32}P]dCTP using Klenow fragment. Binding reactions contained 10 μg nuclear extract and 10 000–50 000 counts/min radiolabelled oligonucleotide in 1 mM Tris–HCl, 50 mM KCl, 1 mM EDTA and 5% glycerol. Specific binding was confirmed by competition experiments with a 50-fold excess of unlabelled, identical oligonucleotides or cAMP response element-containing oligonucleotides. For antibody supershift assays, the reactions were incubated for 20 min with 1 μg antibody after the addition of radiolabelled probes. Binding reactions were electrophoresed in 4% native polyacrylamide gels using 0·5 × Tris–Borate–EDTA buffer followed by autoradiography.

Cytokine assay

The quantities of IL-12 p70 and IL-6 in the culture supernatants were determined via sandwich ELISA using mAbs specific for each cytokine, as described previously.[16] The mAbs used to coat the plates and the biotinylated second mAb were as follows: for IL-12 p70, C18.2 and C17.8; for IL-6, 20F3.11 and 32C11.4. Standard curves were generated using recombinant cytokines (BD Pharmingen, San Jose, CA; CABD Pharmingen, San Jose, CA). The lower limits of detection were 15·625 pg/ml for IL-12p70 and 39·06 pg/ml for IL-6, respectively.

Statistical analysis

The data are expressed as the means ± standard deviation, as indicated by bars in the figures. Student's *t*-test was used to determine the statistical differences between various experimental and control groups. A *P*-value of $< 0{\cdot}05$ was regarded as significant.

Results

AIMP1 up-regulates the expression of TLR2 in BM-DCs

To determine whether AIMP1 is capable of regulating the expression of TLRs, BM-DCs were treated with AIMP1 (0–500 nM) for various times and the expression levels of TLRs were determined by reverse transcription-PCR. Expression of TLR1, -2, -3 and -7 was highly induced by AIMP1 treatment in BM-DCs, whereas the expression of TLR4, TLR6 and other TLRs was down-regulated or remained unchanged (Fig. 1a and see Supplementary material, Fig. S2). As TLR2 and TLR4 are potent receptors that contain pathogen-associated molecular pattern in BM-DCs, we further investigated expression levels of TLR2 and TLR4 followed by AIMP1 treatment. In particular, the expression of TLR2 was up-regulated by AIMP1

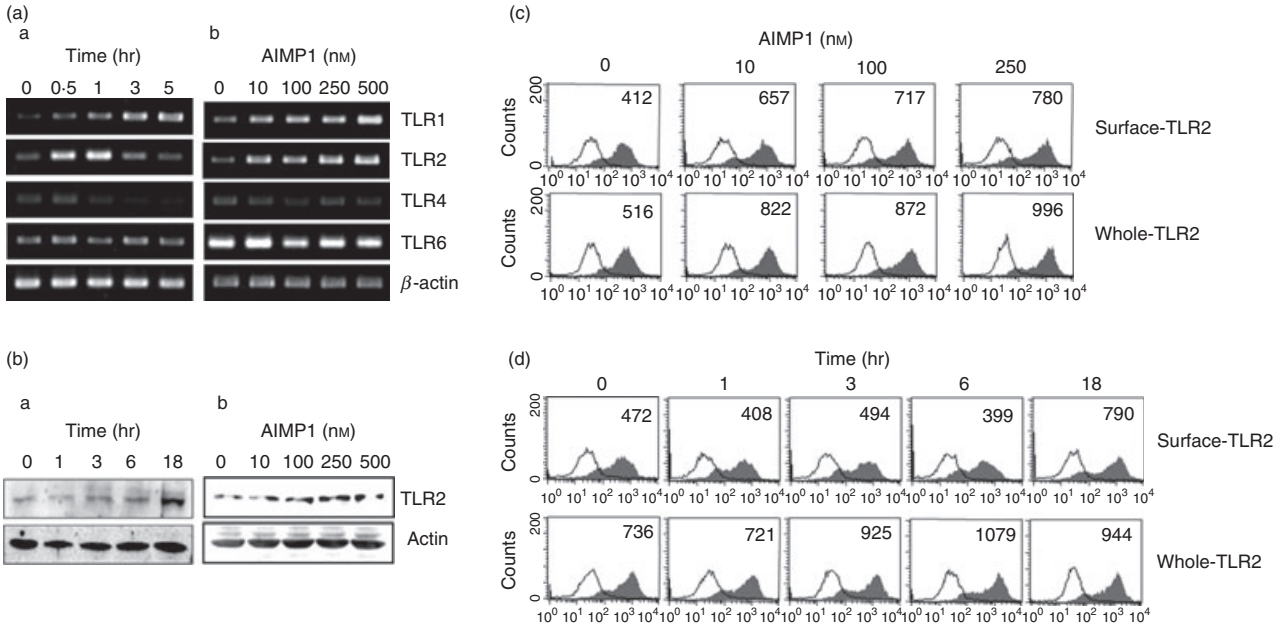

Figure 1. Up-regulated expression of Toll-like receptor 2 (TLR2) by aminoacyl tRNA synthetase-interacting protein 1 (AIMP1) in bone marrow-derived dendritic cells (BM-DCs). The BM-DCs from C57BL/6 mice were generated with granulocyte–macrophage colony-stimulating factor for 6 days. On day 6, bone marrow-derived immature DCs were harvested for further experiments. (a) BM-DCs were treated with AIMP1 (500 nM) for the indicated time or with various concentrations of AIMP1 (0–500 nM) for 1 hr. Total RNA was isolated from the cells and cDNA was prepared by Cyclescript RTase. Reverse transcription-PCR products for TLR2, TLR4, and β-actin were analysed in 1·5% agarose gels. (b) BM-DCs were treated with AIMP1 (500 nM) for the indicated times or with various concentrations of AIMP1 (0–500 nM). Whole cell lysates were prepared from the cells, and immunoblotted with 1 μg/100 μl of anti-TLR2 monoclonal antibody. (c, d) BM-DCs were treated with AIMP1 (0–500 nM) for the indicated times. The expression of TLR2 on the cell surface or on whole cells in $CD11c^+$ cells was determined via cytofluorometric analysis using phycoerythrin-conjugated anti-TLR2 (filled), or with a phycoerythrin-conjugated isotype control monoclonal antibody (open). The values in the histograms represent the mean fluorescence intensity (MFI). The data are representative of three independent experiments.

in a time- and dose-dependent manner, whereas TLR4 expression was not altered or was down-regulated (Fig. 1a). Protein expression of TLR2 in whole cell lysates was increased after 18 hr of stimulation (Fig. 1b). To confirm the increased TLR2 protein levels in the AIMP1-stimulated DCs, we stained the cell surface with phycoerythrin-conjugated anti-TLR2 and analysed via flow cytometry. After 3 hr of stimulation with AIMP1, the whole TLR2 protein levels, which were stained via surface staining and intracellular staining, were increased. TLR2 protein on the cell surface was detected 18 hr after stimulation (Fig. 1d). We also observed that the expression of TLR2 on the surface or whole cells was increased by AIMP1 treatment in a dose-dependent manner (Fig. 1c).

As it was reported that TLR4 ligation can also up-regulate the expression of TLR2,[17] we evaluated the involvement of LPS in the AIMP1-mediated TLR2 expression. To exclude TLR4 signalling on the up-regulated expression of TLR2, we employed the C3H/HeJ mouse, which has a single amino acid substitution in TLR4 making it insensitive to endotoxin.[18] As anticipated, TLR2 expression was up-regulated by AIMP1, but not by LPS in C3H/HeJ mice (Fig. 2a). The up-regulated expression of TLR2 by AIMP1 was not altered in the presence of polymyxin B, an LPS inhibitor (Fig. 2b), suggesting that the effect of AIMP1 on TLR2 expression was not the result of LPS contamination.

NF-κB signalling is involved in the up-regulation of TLR2 expression in AIMP1-stimulated DCs

It has been previously reported that TLR2 expression can be mediated by mitogen-activated protein kinases (MAPKs) and NF-κB signalling.[19] In an effort to determine whether MAPKs and NF-κB signalling are involved in TLR2 gene expression in DCs, immature DCs were treated with MAPKs inhibitors and an NF-κB inhibitor before AIMP1 stimulation. Expression of TLR2 was inhibited by BAY11-7082, an NF-κB inhibitor, whereas pretreatment with MAPK inhibitors did not influence TLR2 expression in AIMP1-stimulated DCs (Fig. 3a). The expression level of TLR2 in AIMP1-stimulated DCs was reduced with increased concentrations of BAY11-7082 (Fig. 3b). These results showed that TLR2 expression was up-regulated by AIMP1 via NF-κB signalling, but not MAPK signalling. Treatment with BAY11-7082 did not affect the expression of β-actin mRNA by AIMP1-stimulated DCs, suggesting that the inhibition of AIMP1-

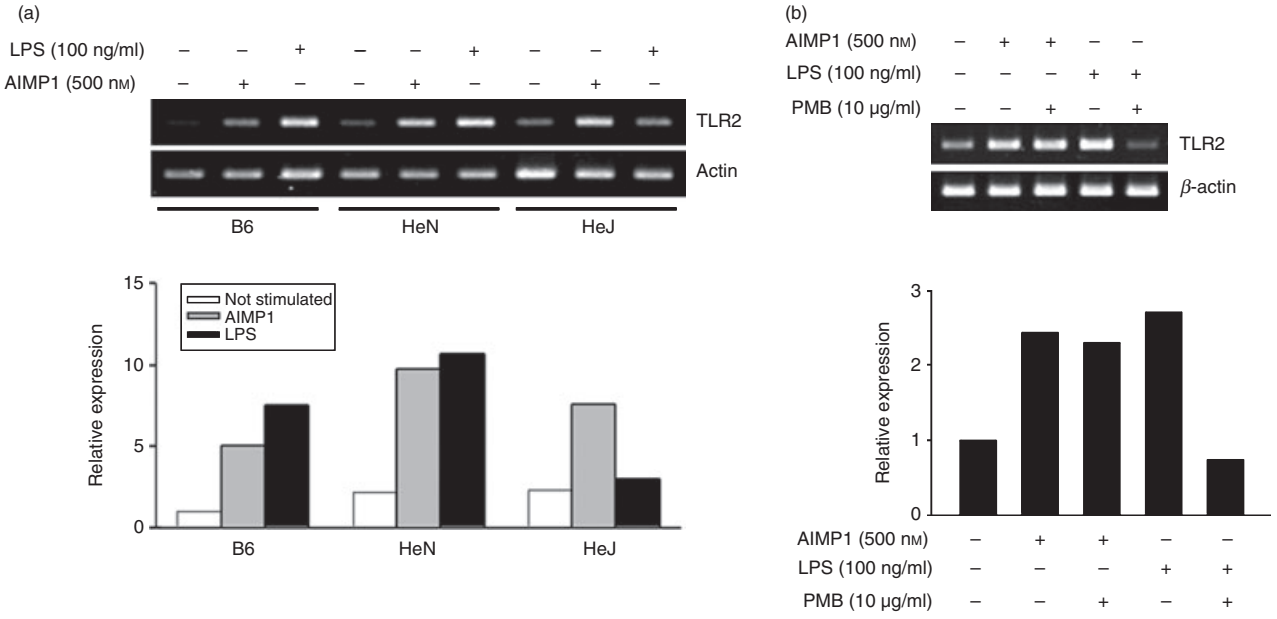

Figure 2. Up-regulated Toll-like receptor 2 (TLR2) expression by aminoacyl tRNA synthetase-interacting protein 1 (AIMP1) was not the result of lipopolysaccharide (LPS) contamination. (a) Bone-marrow-derived dendritic cells (BM-DCs) from different mouse strains (C57BL/6, C3H/HeN and C3H/HeJ) were stimulated with AIMP1 (500 nM) or LPS (100 ng/ml) for 1 hr. (b) BM-DCs were pre-treated for 2 hr with polymyxin B (PMB: 10 μg/ml), followed by 1 hr of additional incubation with either AIMP1 (500 nM) or LPS (100 ng/ml). Total RNA was isolated from the cells and cDNA was prepared via Cyclescript RTase. Reverse transcription-PCR products for TLR2 and β-actin were analysed in 1·5% agarose gels. Similar results were noted in three independent experiments.

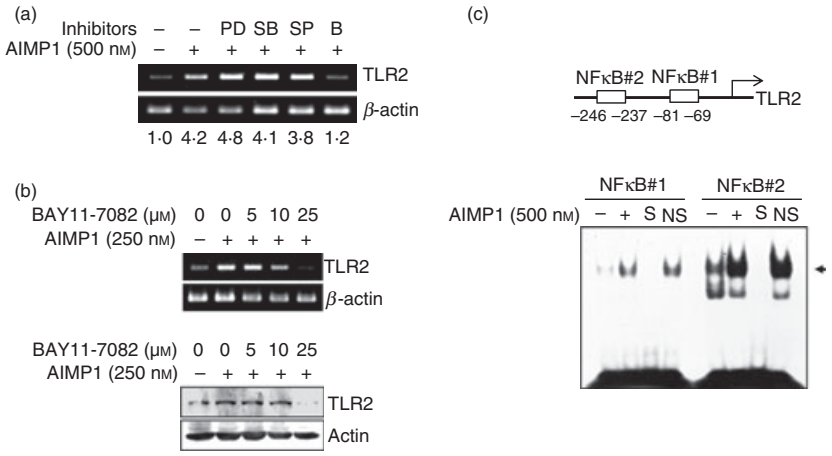

Figure 3. Up-regulated Toll-like receptor 2 (TLR2) expression by aminoacyl tRNA synthetase-interacting protein 1 (AIMP1) was mediated by nuclear factor-κB (NF-κB) signalling, not by mitogen-activated protein kinases. (a) Bone-marrow-derived dendritic cells (BM-DCs) were pre-treated for 1 hr with PD (PD98059, 25 μM), SB (SB203580, 25 μM), SP (SP600125, 25 μM), and B (BAY11-7082, 25 μM). Subsequently, BM-DCs were stimulated with AIMP1 (500 nM) and incubated for 1 hr. The intensity was analysed using Image J Software (provided by NIH Image). (b, upper panel) BM-DCs were pre-treated with the various concentrations of BAY11-7082 (0–25 μM) for 1 hr and further stimulated with AIMP1 (250 nM) for 1 hr. Total RNA was isolated from the cells. Reverse transcription-PCR products for TLR2 and β-actin were analysed in 1·5% agarose gels. (b, lower panel) BM-DCs were pre-treated with various concentrations of BAY11-7082 (0–25 μM), followed by stimulation with AIMP1 (250 nM). Whole cell lysates were prepared from the cells and immunoblotted with anti-TLR2. (c, upper panel) Schematic representation of the NF-κB sites of the mouse TLR2 promoter region are as shown, along with two NF-κB-binding sites. The nucleotide sequence numbers for each of the NF-κB sites are shown. (c, lower panel) Nuclear extracts from AIMP1-stimulated DCs were evaluated for κB-binding activity in the gel shift assay using labelled oligonucleotides containing TLR2-specific κB-binding sites (NF-κB#1 or NF-κB#2), as indicated. The data are representative of three independent experiments.

enhanced TLR2 expression with BAY11-7082 was not the result of generalized impairment of cell metabolisms.

There are two NF-κB binding sites located at the TLR2 promoter region,[20] as depicted schematically (Fig. 3c, upper panel). To determine the effect of AIMP1 on the induction of TLR2–κB function, we analysed the NF-κB binding activity manifesting in the nuclear extracts of either unstimulated DCs or AIMP1-stimulated DCs. As shown in Fig. 3(c), NF-κB binding activity was markedly increased in AIMP1-stimulated DCs, compared with that in the unstimulated DCs. The NF-κB binding activity in AIMP1-stimulated DCs was higher at the NF-κB#2 binding site (−246 to −237) than at the NF-κB#1 binding site (−81 to −69). Specific binding was confirmed by the competition experiments with a 50-fold excess of unlabelled, identical oligonucleotides (S) or cAMP response element-containing oligonucleotides (NS).

AIMP1 enhances LTA-induced or Pam3Cys-induced expression of CD40 molecule on BM-DCs

Activation of TLR2 results in DC activation characterized by the up-regulated expression of cell surface molecules and inflammatory cytokines. Lipoteichoic acid and Pam3Cys were used as TLR2 and TLR1/2 agonists, respectively. To evaluate the effects of AIMP1 on the LTA-induced or Pam3Cys-induced expression of activation markers on BM-DCs, the DCs were simultaneously incubated with AIMP1 and either LTA or Pam3Cys. As shown in Fig. 4, combined stimulation with AIMP1 and either LTA or Pam3Cys additively induced the expression of CD40, although they did not enhance the expression of MHC class II, CD80 and CD86 on DCs. Hence, AIMP1 could augment the TLR2 ligand-induced activation of DCs to enhance the expression of the co-stimulatory molecule, CD40.

AIMP1 enhances LTA-induced cytokine production through TLR2 signalling

We next sought to explore the effect of AIMP1 on TLR2 agonist-induced cytokine production on BM-DCs. To evaluate the enhancing effect of AIMP1 on cytokine production from TLR2 agonist-challenged DCs, the DCs were simultaneously incubated with AIMP1 and either LTA or Pam3Cys. Production of IL-12p70 and IL-6 was additively increased from LTA-stimulated or Pam3Cys-stimulated DCs in the presence of AIMP1 (Fig. 5a,b). Enhanced

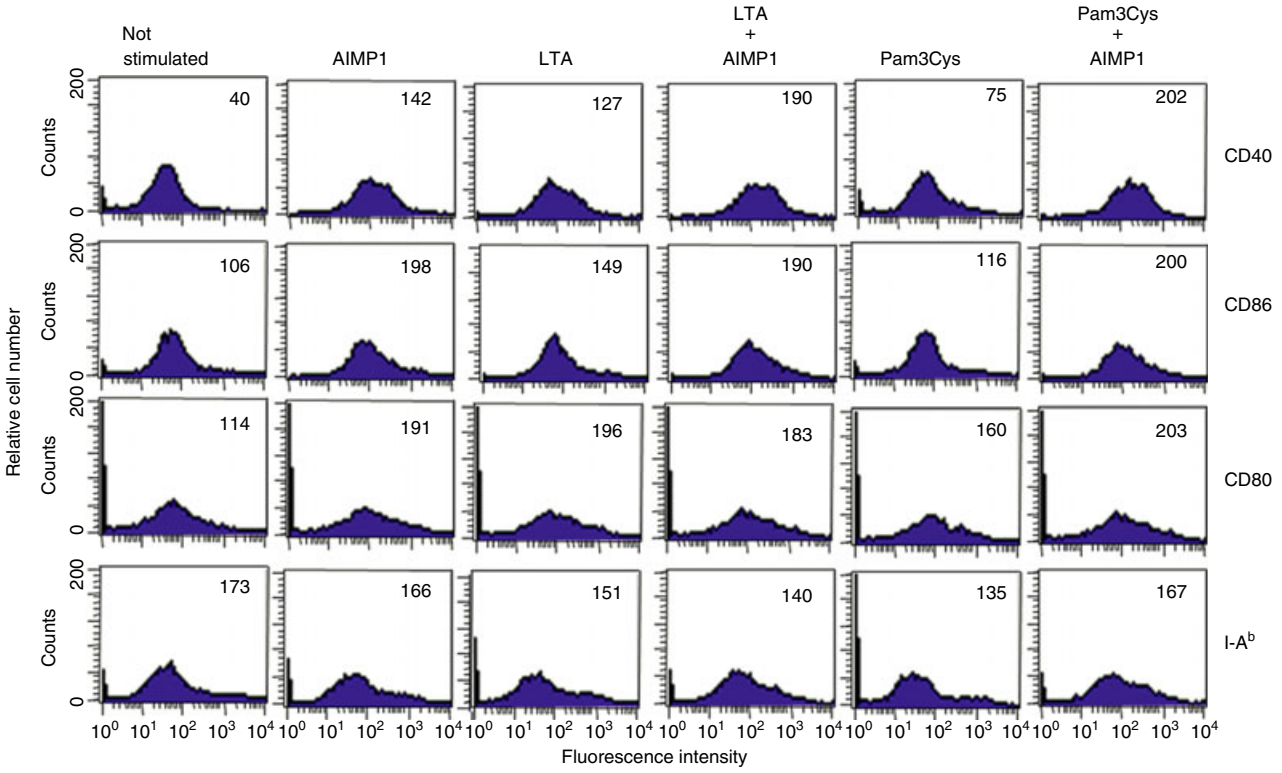

Figure 4. Aminoacyl tRNA synthetase-interacting protein 1 (AIMP1) enhanced expression of lipoteichoic acid (LTA) or Pam3Cys-induced dendritic cell (DC) activation markers. Bone-marrow-derived DCs were stimulated with the indicated concentrations of AIMP1 (250 nM) and LTA (1 μg/ml) or Pam3Cys (0·1 μg/ml). After 18 hr of stimulation, the expression of CD40, I-A^b, CD80 and CD86 molecules on $CD11c^+$ cells was determined via flow cytometric analysis using phycoerythrin-conjugated antibodies. The expression level of activation markers was shown by histogram plot analysis. Mean fluorescence intensity was described in the frames. The data are representative of three independent experiments.

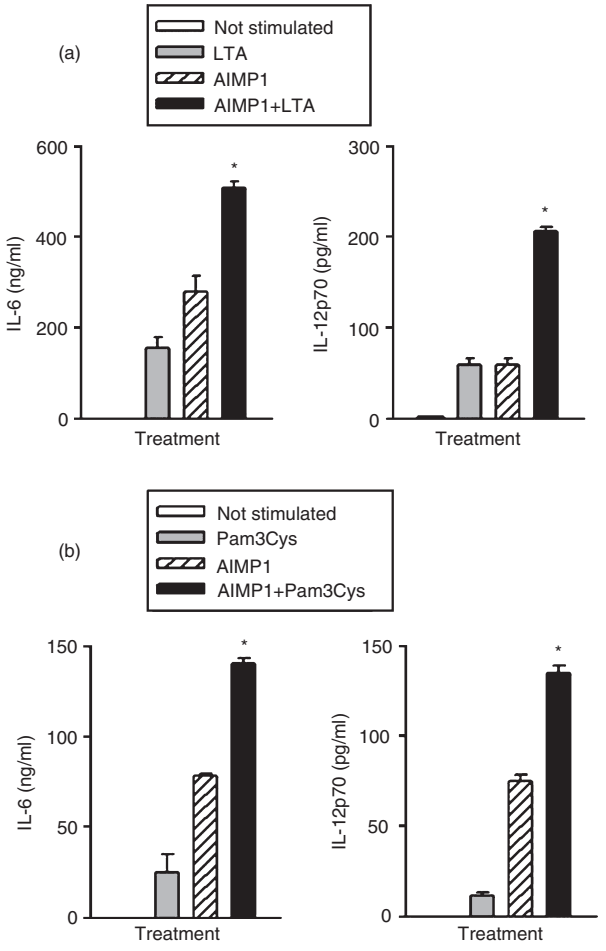

Figure 5. Aminoacyl tRNA synthetase-interacting protein 1 (AIMP1) enhanced the expression of pro-inflammatory cytokines in lipoteichoic acid (LTA) or Pam3Cys-stimulated dendritic cells (DCs). Bone-marrow-derived DCs were simultaneously incubated with both AIMP1 (250 nM) and LTA (1 μg/ml) (a) or Pam3Cys (0·1 μg/ml) (b) for 18 hr. Culture supernatants were collected for interleukin-12p70 (IL-12p70) and IL-6 ELISA. The results are presented as the means ± SEM ($n = 3$). *$P < 0{\cdot}001$, relative to a group of single (AIMP1, LTA or Pam3Cys) -treated group. The data are representative of three independent experiments.

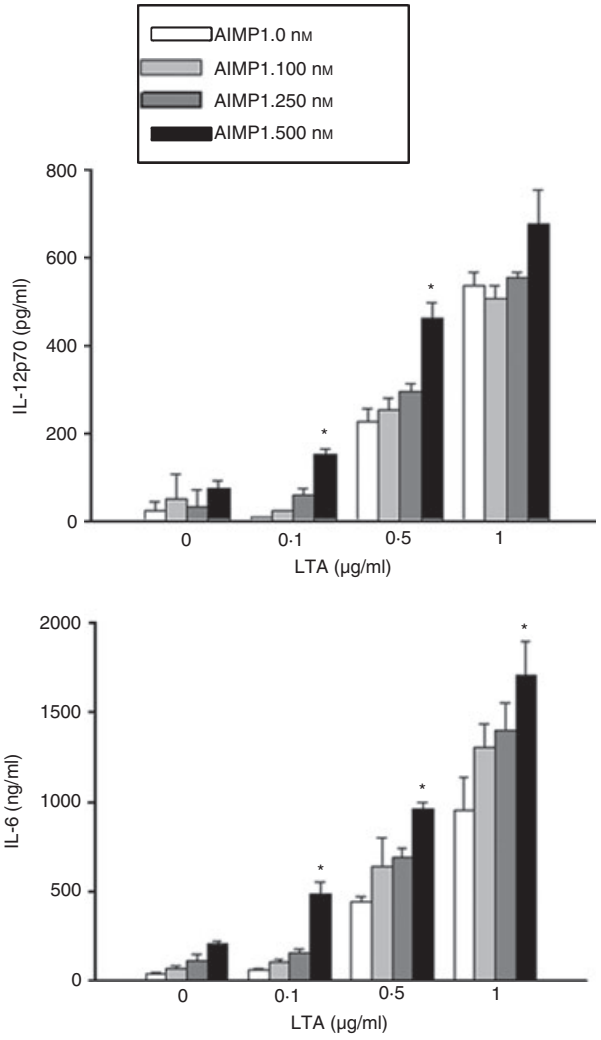

Figure 6. Aminoacyl tRNA synthetase-interacting protein 1 (AIMP1) sensitized and enhanced the expression of pro-inflammatory cytokines in lipoteichoic acid (LTA) or Pam3Cys-stimulated dendritic cells (DCs). Bone-marrow-derived DCs were pre-incubated for 3 hr with AIMP1 (100–500 nM), after which the cells were washed and further incubated for 18 hr with LTA (0·1–1 μg/ml). Culture supernatants were collected for interleukin-12p70 (IL-12p70) and IL-6 ELISA. The results are presented as the means ± SEM ($n = 3$). *$P < 0{\cdot}005$, relative to a group of single (AIMP1or LTA)-treated group. The data are representative of three independent experiments.

cytokine production by AIMP1 treatment was also observed in DCs from C3H/HeJ mice (see Supplementary material, Fig. S3). The expression of TLR2 protein was increased at 18 hr after AIMP1 stimulation, for which reason DCs were pre-incubated for 3 hr with AIMP1, after which the DCs were stimulated with LTA. As expected, enhanced production of IL-12p70 and IL-6 was observed in AIMP-sensitized DCs at the low-dose of LTA (Fig. 6).

We further confirmed any involvement of TLR2 in the enhanced cytokine production by AIMP1 and LTA. The DCs were pre-treated with an anti-TLR2 neutralizing antibody, after which the DCs were treated with AIMP1 and LTA, and cytokine levels in the supernatants were determined via ELISA. As anticipated, the enhanced levels of IL-12p70 and IL-6 by AIMP1 treatment were significantly inhibited by anti-TLR2 or an isotype antibody of anti-TLR2 (Fig. 7a,b). These data indicated that the AIMP-1-mediated enhancement of cytokine production in LTA-stimulated DCs was mediated via TLR2 signalling.

Discussion

Toll-like receptors are well-known receptors that recognize invading pathogens that are constitutively expressed on DCs.[21,22] The TLRs recognize and mediate intracellular

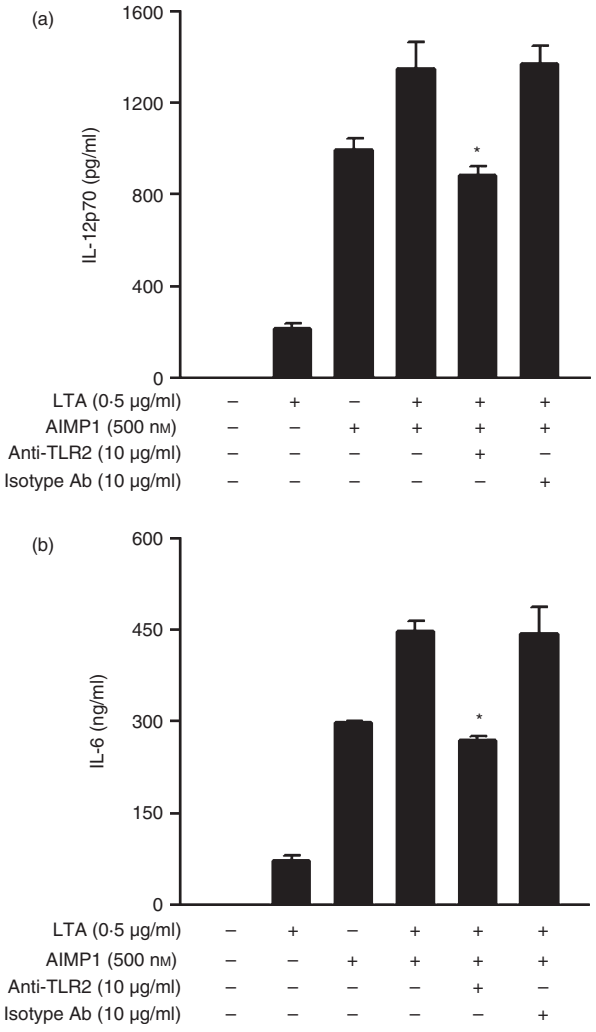

Figure 7. Enhanced cytokine production by aminoacyl tRNA synthetase-interacting protein 1 (AIMP1) in lipoteichoic acid (LTA) or Pam3Cys-stimulated dendritic cells (DCs) was mediated by Toll-like receptor 2 (TLR2) signalling. Bone-marrow-derived (BM-) DCs were pre-incubated for 1 hr with anti-TLR2 (mouse IgG1) or isotype antibody (mouse IgG1). Subsequently, BM-DCs were stimulated for 18 hr with LTA (0·5 μg/ml) in the presence of AIMP1 (500 nM). Culture supernatants were collected for IL-12p70 (a) and IL-6 (b) ELISA. The results are expressed as the means ± SEM ($n = 3$). *$P < 0{\cdot}005$, relative to a group of AIMP1 plus LTA-stimulated DCs. The data are representative of three independent experiments.

signals for a broad range of microbial components. The regulation of the expression level of TLRs is capable of influencing TLR signalling, which can induce the expression of pro-inflammatory genes by antigen-presenting cells. Previously, it has been reported that tumour necrosis factor-α increases TLR2 expression in macrophages.[23] Additionally, tumour necrosis factor-α induces AIMP1 expression and secretion from macrophages, which can form a positive feedback loop with one another, to amplify inflammatory responses to tissue injury.[9] These results raise the question as to whether the tumour necrosis factor-α-induced AIMP1, an endogenous mediator, is capable of regulating the expression of TLRs.

In this study, we focused on the effects of AIMP1 on TLR2 expression and TLR2-mediated immune responses. AIMP1 has been detected in autoimmune disease[24] and acute lung inflammation,[13] in which TLR2 signalling is involved.[25,26] Up-regulated expression of TLR2 by AIMP1 can be a clue to determine the role of AIMP1 in inflammation. We have demonstrated here that AIMP1 up-regulated TLR2 expression, and that this up-regulation is mediated by NF-κB activation. Furthermore, AIMP1 was demonstrated to enhance the production of IL-12 and IL-6, and also to increase the expression of CD40 co-stimulatory molecule in DCs treated with TLR2 agonists.

Both TLR2 and TLR4 recognize their extraceullar ligands. AIMP1 was observed to up-regulate TLR2 expression, but did not affect TLR4 expression. Additionally, because TLR1, TLR2 and TLR6 are closely related on the basis of their amino acid sequences, genomic structures and functions,[27] we measured the expression levels of TLR1 and TLR6. AIMP1 was demonstrated to up-regulate the expression of TLR1, but not TLR6 (Fig. 1a).

AIMP1 was expressed in LPS-induced acute lung inflammation. In lung homogenates, the concentration of AIMP1 is 3 pg/ml and rises to 15 pg/ml after stimulation with LPS.[13] In sera from healthy volunteers and from patients with systemic lupus erythematosus, AIMP1 is likely to circulate at a concentration from < 5 to 30 ng/ml. The concentrations of AIMP1 used in the experiments were higher than the physiological concentrations of AIMP1, because recombinant AIMP1 was purified from *E. coli*, not from mammalian cells, and relatively high doses of AIMP1 have been used in other experiments.[7,9,11] It was known that recombinant protein purified from *E. coli* had reduced bioavailability because of insufficient post-translational modification (e.g. glycosylation).[28] However, the enhancing effects of AIMP1 on TLR2-mediated responses need to be further confirmed using recombinant AIMP1 protein from mammalian cells.

In conclusion, TLR2 activation by LTA after exposure to AIMP1 results in enhanced production of pro-inflammatory cytokines and expression of co-stimulatory molecules on DCs. These results indicate that AIMP1 may enhance the immune responses by regulating the TLR expression.

Acknowledgements

We thank Y. G. Ko and J. H. Kim for helpful discussions and E. P. Cohen for critical review of the manuscript. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (2010-0009243).

Disclosures

No conflict of interest reported.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bone marrow-derived dendritic cells (BM-DCs)were separated and cultured, as described in the Materials and methods.

Figure S2. Bone marrow-derived dendritic cells (BM-DCs) from C57BL/6 mice were generated with granulocyte–macrophage colony-stimulating factor for 6 days.

Figure S3. Bone marrow-derived dendritic cells (BM-DCs) were simultaneously incubated for 18 hr with AIMP1 (250 nM) and LTA (1 μg/ml), after which the levels of interleukin-12p70 (IL-12p70) and IL-7 in the culture supernatants were determined via ELISA.

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